Identification of a cytisine-based EED-EZH2 protein-protein interaction inhibitor against metastasis in triple-negative breast cancer cells

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Supporting Information

Supplementary methods

Peptide synthesis

The EZH2 and FITC-EZH2 peptides (FITC-SMFSSNRQKILERTEILNQEWKQR, SMFSSNRQKILERTEILNQEWKQR) were purchased from China Peptides (IGE biotech, China) and were synthesized and purified using SHIMADZU LCMS-2020. The synthesized peptides were analyzed by reverse-phase high-performance liquid chromatography (HPLC) and assessed by mass spectrometry (MS). The purity of all the peptides was greater than 90%. N-terminal or C-terminal fluorescein isothiocyanate (FITC)-labeled EZH2 peptide was used as tracer peptides and the concentrations were determined on the basis of their molecular weights.

CD44+/CD24- staining analysis

MDA-MB-231 cells were seeded in 100 cm^2 dish at a density of 1×10^5 / mL, then treated with **1** or **21** (5 μM) or DMSO control for 12 h. Cells were collected and washed with PBS, followed by incubation for 30 min at 4 °C with FITC- and APC-conjugated anti-mouse IgG or FITC-conjugated anti-CD44 and APC-conjugated anti-CD24 antibodies. Cells were analyzed by flow cytometry using a BD LSR Fortessa Flow Cytometer.

3D cell sphere formation assay

MDA-MB-231 cells (1×10^4) were seeded in Nunclon™ Sphera™ Dishes (Thermo Fisher Scientific) cultured with DMEM/F12 medium, B27 (1:50, Gibco), human recombinant epidermal growth factor (20 ng/mL, Gibco), and basic fibroblast growth factor (20 ng/mL, Gibco). Cells were incubated with **1** (10 or 30 μM) or DMSO control for two weeks, and 3D cell spheres were observed using confocal laser scanning microscopy. The volume of spheres was quantified by Image J software.

Western blotting

MDA-MB-231 cells were seeded in a 6-well plate with a density of 1×10^5 / mL incubated at 37 °C, 5% CO_2 incubator. The western blot assay was performed as the previous report [27]. The antibodies against EED (1:1000), EZH2 (1:1000), H3K27Me3 (1:1000), and β-actin (1:1000) purchased from Abcam (Cambridge, MA, USA), E-cadherin (1:1000), Snail (1:1000), ALDH1A1 (1:1000), CD44 (1:1000), and CD133 (1:1000) purchased from Absin Bioscience (Shanghai, China). Proteins bands were detected by the enhanced chemiluminescent method and the relative band intensity was analyzed by Image Lab.

Statistical analysis

All statistical tests were performed using GraphPad Prism version 8.0 (Graph Pad, San Diego, CA, USA). Statistical significance was determined using the Student's *t*-test for experiments comparing two groups. Comparisons among groups were analyzed using analysis of variance (ANOVA). Unless stated otherwise, *p* values were 2-tailed and considered significant if $P < 0.05$. Error bars represent SEM of three experiments unless stated otherwise.

Table S1 Promoters of qPCR and ChIP-qPCR primers used in this paper.

Promoter's name	Forward primer	Reverse primer
snail1-RT	5'-CCCAGTGCCTCGACCACTAT-3'	5'-GCTGGAAGGTAAACTCTGGATTAGA-3
ALDH1A1	5'- GCACGCCAGACTTACCTGTC -3'	5'- CCTCCTCAGTTGCAGGATTAAAG -3'
β-actin-RT	5'-AGGCACCAGGGCGTGAT-3'	5'-CTCTTGCTCTGGGCCTCGT-3'
Snail-CHIP	5'-TGTTCAGGGCTGTGTAGAC-3'	5'-GAGCTGCTGACCTTTGG-3'
ALDH1A1-CHIP	5'- TGGCACTGGTTATTCAACGTGGTC-3'	5'- GAGGGTGGAAGCTCTTGTAGGTTT-3'
β-actin-CHIP	5'-CGGCGCCCTATAAAACCCA-3'	5'-CTGGCCGGGCTTACCTGG-3'

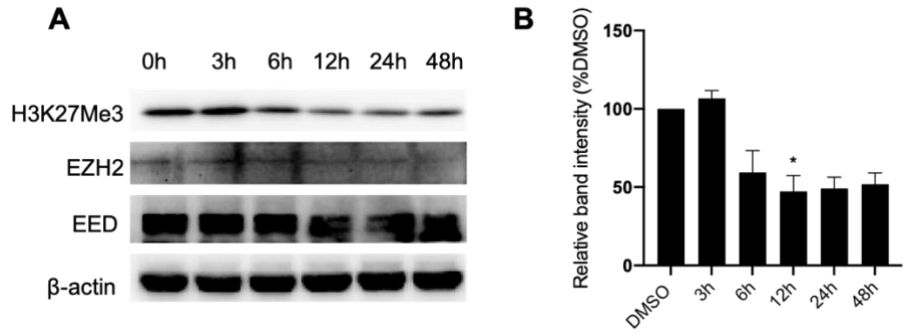

Figure S1. Compound **1** achieved optimal inhibition activity at 12 h. (A) Inhibition of H3K27Me3 protein levels at different time points. (B) Relative densitometry analysis of H3K27Me3. Data are represented as mean ± SD. Student's t test, * $p < 0.05$.

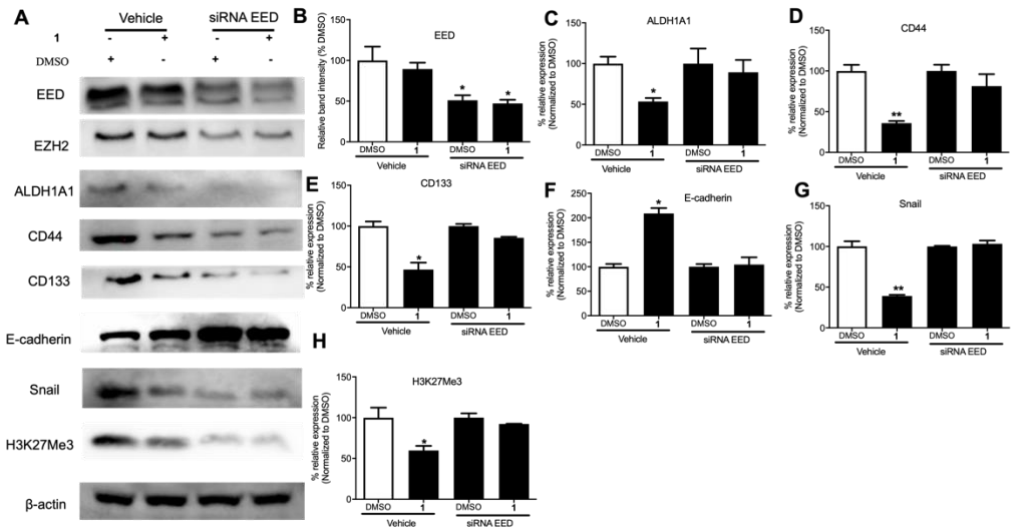

Figure S2. EZH2 is the direct target of compound **1**. (A) EED siRNA was transformed into MDA-MB-231 cells. EED, EZH2, ALDH1A1, CD44, CD133, E-cadherin, Snail and H3K27Me3 were detected by Western blotting with β-actin as loading control. (B-H) Relative densitometry analysis. Student's t test, * $p < 0.05$, ** $p < 0.01$.

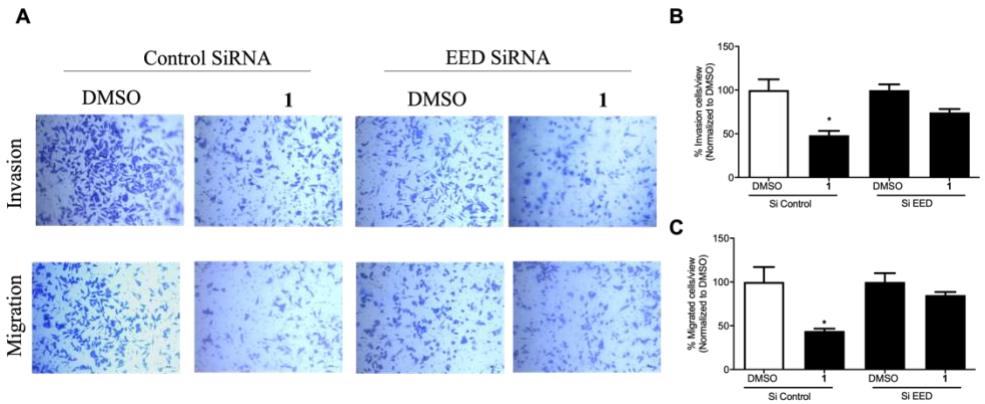

Figure S3. The anti-migration and anti-invasion effect of **1** in EED knockdown cells. (A) EED siRNA treatment produced efficient target knockdown in MDA-MB-231 cells. Cells were examined for their migration and invasion ability after treatment of **1**. (B-C) Quantitative analysis of cellular migration and invasion. Student's t test, * $p < 0.05$.

Reference

[1] G.J. Yang, W. Wang, P.M. Lei, C.H. Leung, D.L. Ma, A 7-methoxybicoumarin derivative selectively inhibits BRD4 BD2 for anti-melanoma therapy, Int. J. Biol. Macromol. 164 (2020) 3204-3220.